

Biology Ph.D. School, Programme Immunology
Eötvös Loránd University of Arts and Science, Faculty of Natural Sciences
Head of Ph.D. School and Ph.D. Programme: Prof. Anna Erdei

**Epigenetic analysis of the regulatory regions of latent Epstein-
Barr virus genomes in lymphoid and nasopharyngeal carcinoma
cell lines**

Ph.D. Thesis

Anita Varga-Koroknai

Place of research: National Centre for Epidemiology,
Microbiological Research Group

Supervisor: Dr. János Minárovits, M.D., Ph.D., Doctor of MTA,
Head of the research group

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INTRODUCTION

One of the most important tools of gene expression control is the regulation of gene transcription, which can be achieved by based on post-synthetic modifications of the genome and protein-DNA interactions. The dynamic structure of chromatin is a central player in this regulation, because only a precise, coordinated opening and closing of chromatin can assure the utilization of genetic information at the suitable location and time. The most important epigenetic regulatory mechanisms are DNA methylation and the modifications of the tails of the DNA-contacting histones. Histone modifications – e.g. acetylations and methylations – can regulate gene expression through altering the chromatin structure.

Epstein-Barr virus (EBV) is a suitable model to investigate the complex system regulating transcription, because its 172 kbp long genome persists in chromosome-like episomal form during the latent infection, and similarly to the chromosomal DNA, replicates only once during the cell cycle. EBV is a human herpesvirus, which infects epithelial cells and B cells, respectively. The viral gene expression and promoter usage depend on the latency type. Out of six EBV encoded nuclear antigens (EBNAs) only the EBNA1 protein is expressed from a transcript initiated at the Q-promoter (Qp) in latency type I, while the C-promoter (Cp) and the promoters coding membrane proteins (LMP1 and LMP2) are silenced. In contrast, latency type III is characterized through the expression of all latency genes; proteins EBNA1, -2, -3A, -3B, -3C, -LP are expressed from Cp, and Qp is inactive.

The latent, episomal EBV genomes are anchored to the nuclear matrix via a putative “Locus control” region (LCR). This region extends from the 5’ end of EBER locus to the 3’ end of *oriP* located within the *Bam*HI C fragment of the EBV genome. The putative, “open chromatin” LCR of EBV can be characterized by DNase I hypersensitive sites (HSs), where transcription factors can bind.

Thus, cell lines representing the various latency types of EBV can be used as an optimal experimental system to investigate the chromatin structure and different histone modifications. Because EBV is associated with several human diseases, our results may help to understand not only the regulatory mechanisms necessary to ensure a differential gene expression, but also the working mechanisms of an important human pathogenic virus.

OBJECTIVES

1. Mapping hypersensitive sites in the putative „Locus control” region, C- and Q-promoter, respectively, in cell lines representing the three main latency types of EBV.
2. Determining the activity of latent Cp, Qp and the expression level of LMP2A and LMP1 in EBV carrying cell lines.
3. Examination of the acetylation state of H3 and H4 histones and the level of histone H3K4 dimethylation (H3K4me2) at latent EBV promoters Cp, Qp and LMP2Ap in EBV genomes carried by lymphoid cell lines and a nasopharyngeal carcinoma cell line.
4. Defining the level of histone modifications at different regions (regulatory region, initiation site, coding region) of the LMP1 promoter.
5. Examination of the effect of a histone-deacetylase inhibitor on Cp and LMP2Ap activity and on the level of H4 histone acetylation in cell lines, where the promoters are silent.

MATERIALS AND METHODS

Cell lines:

Well characterized, cell lines carrying latent EBV genomes and corresponding to latency type I, II and III were used in this study. EBV latency type I was represented by Burkitt's lymphoma (BL) cell lines: Mutu-BL-I-CI-216 and Rael, whereas latency type III by a lymphoblastoid cell line CB-M1-Ral-STO and a BL line Mutu-BL-III-CI-99 were used. Mutu-III was generated during a spontaneous latency type switch of *in vitro* cultivated latency type I Mutu clones. The type III lymphoblastoid cell line (LCL) called CB-M1-Ral-STO was immortalized by the virus strain from Rael. Latency type II was represented by the nasopharyngeal carcinoma derived cell line C666-1 of epithelial character.

DNase I hypersensitivity assay:

DNase I hypersensitive sites (HSs) can be mapped with a method called DNase I hypersensitivity assay with a precision of +/- 100 bp. During this method first nuclei were

isolated from different cell lines. The isolated nuclei were digested using increasing concentrations of DNase I. The DNA isolated from DNase I treated and control nuclei were digested with appropriate restriction enzymes, and analyzed by Southern-blotting with hybridizations probes labeled with a highly sensitive, non-radioactive Digoxigenin (DIG). The probes were labeled with DIG-dUTP by PCR labeling procedures. (See the protocol in DIG Application Manual, Roche)

Isolation of RNA and RT-PCR:

RNA was isolated from the cell lines described above using TRI Reagent (Sigma), and then cDNA was synthesized with a reverse transcriptase SuperScript III (Invitrogen) and promoter specific primers, according to the manufacturer's protocol.

Real-time PCR:

The relative DNA concentration of the samples can be determined with a quantitative method called Real-time PCR. Using cDNA template I applied this method for expression level examination, and using chromatin immunoprecipitated DNA I applied it to analyze the level of histone acetylation and methylation.

For Real-time PCR I used the LightCycler FastStart DNA Master SYBR Green I kit (Roche). We experimentally adjusted the conditions for the PCR reaction, taking the recommendation of the technical bulletin into consideration. SYBR Green binds to double stranded DNA and its surplus energy manifesting in photons can be detected by measurement of the fluorescence signal at 530 nm. The amount of bound SYBR Green and therefore the magnitude of the detected signal is proportional to the length and amount of double stranded DNA. Because SYBR Green can bind to any double stranded DNA, to prove that only the desired PCR product has been amplified, I performed a melting curve analysis and checked the PCR product with agarose gel-electrophoresis as well in each cases.

Chromatin Immunoprecipitation (ChIP) assay:

The ChIP protocol I used is identical with the method of Farnham at al. published in 2002, with slight modifications.

With the method of Chromatin Immunoprecipitation we can detect protein-DNA interactions *in vivo*. First protein-DNA interactions are fixed with formaldehyde in the cells, then the isolated, sonicated chromatin (500-1000 bp) is immunoprecipitated using specific antibodies directed to modified histones and Protein A-agarose beads binding to the Fc part of the antibodies. The precipitate will contain the DNA fragments bound to the proteins of interest. Thereafter one can assess in a quantitative manner the ratios of the DNA-fragment bound proteins in different cell lines. Thus using Real-time PCR after ChIP I could determine the relative levels of modified histones (acetylated H3 and H4, lysine 4 methylated H3) in different cell lines.

Trichostatin A treatment:

Trichostatin A (TSA) is an effective inhibitor of histone-deacetylases. TSA treatment results a higher level of histone acetylation that induces gene expression in acetylation-sensitive genes. To make sure, that the higher transcription activity is caused by the higher level of histone acetylation, and not any other transactivating mechanisms, we examined samples treated with a protein synthesis inhibitor cycloheximid (CHX) and another samples treated with both TSA and CHX, respectively. Then chromatin and RNA were isolated from the treated cells.

RESULTS

- 1) Using DNase I hypersensitivity assay, altogether 35 common and unique hypersensitive sites (HSs) were found in the putative “Locus control” region (LCR) and Cp of EBV. The unique HSs were not latency type specific they can be characterised, instead as cell types specific HSs.
- 2) In latency I cell lines a nucleosomal pattern was observed in a region of Cp, which correlates well with Cp inactivity detected in these cell lines.
- 3) In contrast to the results I got at the LCR, there were 8 latency type specific hypersensitive sites at Qp, which fits well to the Qp activity and the presence of the activating histone modifications, too.

- 4) The activity of the latent promoters was in accordance with a published results, i. e. Cp, LMP1p and LMP2Ap were inactive, but Qp was active in latency type I cells, whereas a complementary pattern is observed in latency type III.
- 5) Qp was highly active but the alternative viral promoter Cp was almost silent in the nasopharyngeal carcinoma cell line C666-1, that has been less investigated and characterized as latency type II in the literature. There was a low expression level of LMP1 and LMP2A. Thus, in our hands, the expression pattern of C666-1 was similar to latency type I, instead of latency type II.
- 6) According to the histone modification analysis, we concluded that active Cp is highly acetylated and show a high level of H3K4me2, whereas the silent C-promoters were poor these modified histones with the exception of the Mutu-I clone, where the H3K4me2 level was high. We suggest that a high level of H3K4me2 may contribute to the maintenance of a “poised” chromatin state at the silent Cp in Mutu-I.
- 7) The histone-deacetylase inhibitor TSA induced a significant Cp initiated transcription in the latency type I (Cp off) cell Mutu-I but only slightly in Rael. In addition, treating Mutu-I and C666-1 cells with TSA resulted in an enrichment of AcH4 at Cp.
- 8) We found elevated levels of AcH3, AcH4 and H3K4me2 at the active Q-promoters. Among the cells actively using Qp the highest levels of these histone modifications were observed in C666-1 cells. This corresponds to the high level of Qp initiated transcripts detected in these cells.
- 9) Active LMP2Ap was enriched in AcH3, AcH4 and H3K4me2 in lymphoid cell lines. Regarding the two BLs with low LMP2A expression level TSA and a combination of CHX activated LMP2Ap in Mutu-I but not in Rael, whereas TSA resulted in an increased level of AcH4 in both cell lines. These results show the importance of the combinatorial effects of DNA methylation, histone acetylation, and H3K4me2 on the regulation of LMP2Ap, as LMP2A could be induced with TSA only in Mutu-I cells, containing a broad DNA region without methylated cytosines and moderate levels of H3K4me2, as opposed to the Rael cells, containing patches of highly methylated cytosines and low levels of H3K4me2 at LMP2Ap. This suggests that the DNA

methylation level of this region may affect the level of transcriptional initiation induced by an enrichment of acetylated histones. The moderate increasing H3K4me2 (which may correspond to a more open chromatin structures) may also contribute to better TSA inducibility.

10) Surprisingly, we detected high levels of Ach3, Ach4 and H3K4me2 at LMP2Ap in nasopharyngeal carcinoma cell line C666-1 expressing low levels of LMP2A mRNA. We hypothesized, based on these contradictory results that the patch of highly methylated CpGs at the transcription initiation site in C666-1 cells at LMP2Ap may overcome the activating effects of the high levels of histone acetylation and H3K4 dimethylation.

11) Active LMP1-promoters were highly enriched in Ach3, Ach4 and H3K4me2 in all examined regions (regulatory region, initiation site, coding region).

DISCUSSION

According to the results of the DNase I hypersensitivity assay, I concluded that the putative „Locus control” region of EBV has an opened chromatin structure independently of the latency type. There aren't any latency type specific characteristics of the chromatin structure in the Cp region, except for the nucleosomal pattern observed in latency type I (Cp off) cell lines. In contrast, the Q-promoter has an open chromatin structure in cell lines actively using Qp, whereas the chromatin is closed the case of cell lines with silent Qp. This is further supported by the presence of activating histone modifications observed at active Q-promoters.

In most cases, the examined activating histone modifications (acetylations and H3K4 dimethylation) correlate well with the activity of EBV promoters, suggesting that they play an important role in the regulation of the latent EBV promoters. Analysis of these histone modifications and the results of TSA treatment show that DNA methylation and histone deacetylation act in concert to inhibit transcription, and that the methylation of certain CpGs is prominently important in the regulatory process.

PUBLICATIONS IN CONNECTION WITH THE THESIS

György Fejér*, *Anita Koroknai**, Ferenc Banati, Ildiko Györy, Daniel Salamon, Hans Wolf, Hans Helmut Niller, and Janos Minarovits. „**Latency type-specific distribution of epigenetic marks at the alternative promoters Cp and Qp of Epstein-Barr virus**”. Journal of General Virology (2008); 89: 1364-1370

Borbala Gerle*, *Anita Koroknai**, György Fejér, Agnes Bakos, Ferenc Banati, Kalman Szenthe, Hans Wolf, Hans Helmut Niller, Janos Minarovits, and Daniel Salamon. „**Acetylated Histone H3 and H4 Mark the Upregulated LMP2A-Promoter of Epstein-Barr Virus in Lymphoid Cells**”. Journal of Virology (2007); 81 (23): 13242-13247.

Agnes Bakos, Ferenc Banati, *Anita Koroknai*, Maria Takacs, Daniel Salamon, Susanna Minarovits-Kormuta, Fritz Schwarzmann, Hans Wolf, Hans Helmut Niller, Janos Minarovits.: „**High resolution analysis of CpG methylation and in vivo protein-DNA interactions at the alternative Epstein-Barr virus latency promoters Qp and Cp in the nasopharyngeal carcinoma cell line C666-1**”. Virus Genes (2007); 35: 195-202.

Presentations in connection with the results:

Anita Koroknai, Ferenc Bánáti, György Fejér, Hans Helmut Niller, Dániel Salamon, János Minárovits. „**Analysis of histone H3 and H4 acetylation and histone H3-K4 methylation at the latent EBV promoter LMP-1**”. 15th International Congress of the Hungarian Society for Microbiology, Budapest, 2007

Anita Koroknai, János Minárovits, Fritz Schwarzmann, Hans Helmut Niller. „**Mapping of DNase I hypersensitive sites (HS) in the putative locus control region of latent Epstein-Barr virus**”. 1st Central European Forum for Microbiology, Keszthely, 2005

Borbála Gerle, *Anita Koroknai*, Ferenc Bánáti, György Fejér, Dániel Salamon, János Minárovits. „**Analysis of histone H3 and H4 acetylation and histone H3-K4 methylation at the latent EBV promoter LMP2A**”. 1st Central European Forum for Microbiology, Keszthely, 2005

Hans Helmut Niller, Dániel Salamon, *Anita Koroknai*, Ferenc Bánáti, György Fejér, Ildikó Győry, Fritz Schwarzmann, Hans Wolf, János Minárovits. „**The locus control region of Epstein-Barr virus**”. 1st Central European Forum for Microbiology, Keszthely, 2005